Field Activity and Storage Stability of *Anagrapha falcifera* Nucleopolyhedrovirus (*Af*MNPV) in Spray-Dried Lignin-Based Formulations

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ABSTRACT A multiple-embedded nucleopolyhedrovirus isolated from *Anagrapha falcifera* (Kirby) (*Af*MNPV) has potential to be developed into a microbial bioinsecticide because the host range includes several economic pests. We tested spray-dried *Af*MNPV formulations after storage for insecticidal activity based on bioassays with neonate *Trichoplusia ni* (Hübner). Eight experimental lignin-based spray-dried formulations, a glycerin-based formulation, and an unformulated sample were made with virus stock from three commercial production lots. Samples of these formulations were stored at 30°C in individually sealed sample containers for destructive sampling after 1, 3, and 6 mo whereas the remaining product was stored in glass jars under refrigeration for up to 30 mo. Spray drying did not significantly reduce the initial LC_{50}s of *Af*MNPV in experimental formulations compared with unformulated virus that was not spray dried. Refrigerated storage for 6 mo did not significantly lower virus activity of formulated samples compared with the unformulated *Af*MNPV stored frozen, while samples stored for 30 mo had higher LC_{50} values determined by both droplet and leaf feeding assays. When stored at 30°C, most formulations (22 of 24) maintained insecticidal activity for 3 mo, but most (21 of 24) lost significant activity after 6 mo of storage. The glycerin-based formulation also lost activity within 6 mo of storage at 30°C when compared with frozen unformulated virus, but did not lose activity when stored refrigerated for up to 30 mo. These formulations were evaluated after 7 mo at 4°C for residual insecticidal activity when applied to field grown cabbage. Insecticidal activity was determined against *T. ni* neonates for treated leaf samples collected at 3, 7, 27, and 51 h after application of 2.5×10^{12} obs/ha. Field tests showed no differences in activity among samples of stored formulations and one freshly made formulation. Spray-dried formulations had significantly higher insecticidal activity (67.5% mortality) compared with the unformulated treatment (30% mortality) sampled 3 h after application. At 3, 7, and 27 h after application, the spray-dried formulations had higher residual activity (67%, 59%, and 42% mortality, respectively), compared with the commercial glycerin-based formulation (61%, 38%, and 23% mortality, respectively). These experiments demonstrated that *Af*MNPV in lignin-based spray-dried formulations had a shelf-life of up to 3 mo at 30°C and up to 30 mo at 4°C, and with longer residual insecticidal activity in the field compared with unformulated or a glycerin formulation.

KEY WORDS *Anagrapha falcifera* nuclear polyhedrovirus, shelf-life, spray dry, lignin, microencapsulation

BACULOVIRUSES OCCUR NATURALLY in insects and are capable of causing spectacular epizootics that greatly reduce populations of susceptible insects. Observations of natural control by baculoviruses have led to commercial attempts to mass produce them as biological alternatives to chemical pesticides. However, commercial success stories of virus-based biological insecticides have been few because several economic factors continue to limit commercial development. Recent literature describes characteristics that are required for microbial pesticides to be commercially viable (Lacey and Goettel 1995, Vail et al. 1999, Lacey et al. 2001). These characteristics include an economic host range, low-cost production, efficacious control, and adequate storage stability. All of these characteristics relate to the economics of the product. Typically, baculoviruses have a narrow host range that limits the potential market size of the product when compared with broad spectrum products such as *Bacillus thuringiensis* and chemical pesticides. As a result, companies may not be able to justify the expense of pes-

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ticide registration for a product with limited potential for economic returns. Also, techniques remain expensive for in vivo virus production because of labor costs, and for in vitro virus production because of cell culture media costs. Pest control efficacy in the field is often compromised because of the rapid loss of insecticidal activity resulting from exposure to sunlight (Wood and Granados 1991, Huber and Ludcke 1996). Finally, baculoviruses have been shown to lose activity when stored as a dry preparation for extended periods of time (Lewis and Rollinson 1978, Kaupp and Ebling 1993). All of these characteristics combine to raise the cost to benefit ratio for baculovirus insecticides and thus baculoviruses account for <0.1% of operational pest control in highly industrialized nations (Federici 1999). Improvements related to these characteristics are easy to describe but difficult to obtain.

Anagrapha falcifera nucleopolyhedrovirus (*Af*MNPV), a variant of *Autographa californica* MNPV (Chen et al. 1996, Federici and Hice 1997), was selected for this research because of its activity against a wide range of lepidopteran pests of food and fiber crops (Vail et al. 1996) increasing its potential for commercialization. Also, Certis USA (Columbia, MD) has developed commercial production techniques for this virus, addressing a second limitation to commercialization. As with many other microbial agents, NPVs are easily inactivated by direct solar radiation (Shapiro and Bell 1984, Ignoffo and Garcia 1992, Huber and Ludcke 1996). Addressing this problem, Tamez-Guerra et al. (2000) and McGuire et al. (2001) demonstrated extended field residual activity of lignin-based formulations of baculoviruses (Shasha et al. 1998).

The purpose of this study was to determine shelf life of several lignin-based, spray-dried formulations of *Af*MNPV. Results from these experiments should help to identify important criteria for prolonging activity of *Af*MNPV during storage of formulations known to preserve residual activity in the field. Because the same formulations were produced using three commercial lots of virus, information about the repeatability of formulation production was examined.

Materials and Methods

Insect Colony. Neonate *Trichoplusia ni* (Hübner) from a colony maintained on artificial diet (wheatgerm, Vanderzant et al. 1962) at the USDA–ARS–National Center for Agricultural Utilization Research, Peoria, IL, were used for all bioassays. Founders for this colony were obtained from USDA–ARS–Biological Control Insect Research Laboratory, Columbia, MO, before 1995.

Virus Source. Stocks of the baculovirus originally isolated from celery looper, *Anagrapha falcifera* (Kirby), were provided by Certis USA, Columbia, MD. Virus stocks from three production lots were used to prepare experimental formulations. Production lots were produced using proprietary in vivo techniques. These stocks contained between 1.9 and 5.0 $\times$ 10^9 occlusion bodies per ml (ob/ml). Virus stocks were kept frozen at −20°C until formulated.

Table 1. Production lots of celery looper virus (*Af*MNPV) concentrate provided by Certis USA and used for formulation shelf life experiments

Lot code	Lot #	Production host	OB/mL
A	042798	*T. ni*	3.5×10^9
B	060198	*T. ni*	3.4×10^9
C	060898	*T. ni*	4.3×10^9

Experimental Formulations. Three production lots (identified as lots A, B, and C) of virus (Table 1) were used to prepare each of eight experimental formulations, a glycerin-based formulation, and an unformulated control for a total of 30 formulation $\times$ lot samples. Each virus production lot represented a replication of the formulations. The unformulated treatment was an aqueous dilution of the virus stock at the time of assay. The glycerin formulation consisted of diluting the virus stock to 2×10^9 ob/ml with water, then diluting 1:1 with glycerin for a final concentration of 1×10^9 ob/ml. Each experimental spray-dried formulation was made to contain a standardized 2.2×10^9 ob/g.

Seven formulations were prepared with sodium lignin (PC-1307, Westvaco, Charleston Heights, SC) and various combinations of pregelatinized corn flour (Flour 965, IL Cereal Mills, Paris, IL), titanium dioxide (TiONA, Millennium Inorganic Chemicals, Hunt Valley, MD) and sugar. An eighth formulation was made with potassium lignin (McGuire et al. 2001), two corn flours (Flour 965 and nixamalized corn flour, Guadalupe, N. L. Mexico) and sugar. Calcium chloride was added to all mixtures before spray drying as a cross-linking agent (see Table 2 for formulation compositions).

All spray-dryer feed-stocks for these formulations were standardized to contain 5% wt:vol solids and 2.2×10^9 ob/g of solids. Lignin (PC-1307) was mixed in water at 10% wt:vol for 20 min using a blender (Waring, New Hartford, CT). After the lignin dissolved, the pH was adjusted to 9.0 ± 0.2 with 2% vol:vol sulfuric acid, generally ≈1% of the total volume and was not included in the mixing calculations. The remaining dry ingredients such as flour, sugar, and/or TiO_2 were added to water separately to make a homogenous suspension and then added to the lignin solution followed by the virus stock. Finally, the calcium chloride solution (10% wt:vol) was added slowly to the mixture while mixing vigorously. The total amount of calcium chloride added was 10% wt:wt of the amount of lignin in the mixture. For example, to produce 100 g of product, a dryer feed batch of 2,000 ml final volume may have contained 47.6 g lignin, 23.8 g flour, 23.8 g TiO_2, 4.8 g $CaCl_2$, 64.7 ml *Af*MNPV, and ≈20 ml 2% vol:vol H_2SO_4. Virus amounts were variable depending on the ob concentration of the virus stock.

Formulations were spray dried using a Niro Atomizer Spray Dryer (Niro Atomizer, Inc., Columbia, MD). Drying conditions were 115–125°C inlet tem-

Table 2. List of ingredients used to make 50 g of spray-dried formulations for evaluation of storage stability of *Af*MNP

Formulation	Lignin (g)	TiO_2 (g)	PCF[a] (g)	NCF[b] (g)	Sucrose (g)	$CaCl_2 \cdot 2(H_2O)$ (g)
Lignin	45.5					4.5
Lignin + flour	31.2		15.6			3.1
Lignin + TiO_2	31.2	15.6				3.1
Lignin + flour + TiO_2	23.8	11.9	11.9			2.4
Lignin + sugar	31.2				15.6	3.1
Lignin + sugar	31.2				15.6	3.1
Lignin + 2 flour + sugar	23.8		6.0	6.0	11.9	2.4
Lignin + 2 flour + sugar	23.8		6.0	6.0	11.9	2.4
K-Lignin[c] + 2 flour + sugar	23.8		6.0	6.0	11.9	2.4

[a] PCF = pregelatinized corn flour, Flour 965; Illinois Cereal Mills, Paris, IL.
[b] NCF = nixtamalized corn flour, Maseca; Guadalupe, N. L. Mexico
[c] K-lignin made at USDA in Peoria, IL, by mixing 90.0 g of kraft lignin (Westvaco, Charleston Heights, SC) in 200 ml of deionized water and 11.0 g of potassium hydroxide. The mixture was dried under the hood and sieved through a 30-mesh sieve.

perature, 65–75°C outlet temperature, 18–20 ml/min feed rate, and 6.0 kg/cm^2 air pressure. These samples were collected in glass jars and stored in a freezer (−15°C) until sampled for the storage experiment described below.

Shelf Life Determination. For storage, 12 samples (2 g each) of each formulation were placed into individual 30-ml white plastic containers with screw-on tops, which contained a heat sealing liner that was sealed before storage. The glycerin formulation was also allocated in 2-g portions for storage in these containers. All samples were placed in a dark incubator (Conviron 124L, Pembina, ND) at 30°C. One container (destructive sampling) for each formulation was used to determine LC_{50} and/or moisture content, as indicated below. Optimally, samples were intended to provide monthly evaluations for 1 yr. However, assays of stored samples were terminated after 6 mo of storage because the dried formulations lost activity and this loss was considered sufficient to demonstrate the effect of storage conditions on the formulations. Remaining product for each of the formulations was stored in glass containers for extended storage periods at 4°C. Refrigerated product was sampled after 24 and 30 mo of storage for insecticidal activity using the droplet-feeding assay and at 30 mo with the cotton-leaf assay (below).

Assays for Insecticidal Activity. *Droplet-Feeding Assay.* The droplet-feeding assay used procedures reported by Behle et al. (2000) (modified from Hughes and Wood 1981) to determine an LC_{50} for each formulation sample based on five virus concentrations (3× dilutions). Each virus sample was mixed at 1.0×10^6 ob/ml in a solution containing 2% wt:vol sucrose, 0.1% wt:vol FD&C Blue 1 (Hilton Davis, Cincinnati, OH) and 0.3% wt:vol sodium carbonate. Then, the five concentrations for each sample were made by serial dilution with the blue sucrose/carbonate solution. Sodium carbonate was added to break down spray-dried lignin particles to minimize virus settling from the drops (Tamez-Guerra et al. 2000). Virus concentrations for individual formulations were adjusted as needed to account for the loss of insecticidal activity to provide data suitable for dose–response analysis. For each sample × concentration, ≈60 small drops were placed in a plastic Petri dish and ≈50 neonate *T. ni* were placed in the dish to feed from the drops. After feeding for ≈10 min, 30 larvae that fed, as evidenced by blue-stained gut, were transferred to individual 28-ml clear plastic cups with snap-on caps containing ≈2.5 g artificial diet, and incubated for 7 d at 28°C in a dark Conviron 124-liter incubator. After incubation, live and virus-killed larvae were counted and percentage mortality was calculated for each sample × concentration. Dosage response data were analyzed using POLO-PC (LeOra Software 1987) based on Finney (1971) to determine the LC_{50} for each formulation sample. Experimental formulations were considered significantly different when 90% CL for the LC_{50} did not overlap. These confidence limits were used for comparison because stored samples were evaluated on a specific time schedule and could not be repeated. POLO-PC did not provide confidence limit values when the g parameter exceeded 0.4 for the traditional 95% limits, and 90% limits were used to maintain consistent comparisons throughout this manuscript.

Cotton-Leaf Assay. For the cotton-leaf feeding assay, cotton was grown in a greenhouse for 4–6 wk. Cotton cultivar used for assays of samples stored up to 6 mo was 'DES 119' and for the 30-mo assay was 'DES 607'. The cotton cultivar was changed because of limited seed availability of 'DES 119'. Direct comparisons of these cultivars did not indicate significant differences between the cultivars when used in the following procedure. Leaves from these plants were rinsed with tap water before use in assays. Leaf disks (33-mm diameter) were cut from rinsed cotton leaves and placed individually in plastic 50-mm diameter Petri dishes, each with a filter paper (42.5 mm #1, Whatman International Ltd., Maidstone, UK) to absorb excess moisture. Five dishes were prepared for each treatment concentration. Five concentrations for each formulation were prepared by 3× serial dilutions of virus starting at 1×10^7 ob/ml to 1.2×10^5 ob/ml. Each leaf disk was treated individually with a 100-μl sample concentration, which was spread over the surface of leaf disks with a glass rod and allowed to air dry. Once dried, 10 newly hatched *T. ni* were placed into each dish and incubated at 28°C for a 22–24-h feeding period. After feeding, six live larvae from each dish were transferred to individual cups containing artificial diet,

totaling 30 larvae per treatment × concentration. Percentage mortality was recorded 7 d after larvae were exposed to treated-leaf disks. Mortality data for dosage-response were analyzed using POLO-PC.

Storage Stability. Each formulation was evaluated after 1, 3, and 6 mo of storage with the previously described droplet assay and after 1, 4, and 6 mo using the cotton-leaf assay. For each assay, treatments included the eight formulations × three production lots of virus, with control treatments consisting of the corresponding glycerin formulation, and frozen unformulated virus for each lot of virus. After 6, 24, and 30 mo, the treatments and controls stored at 4°C were also tested for activity.

Additional Statistics. LC_{50} values for each assay data set were subjected to regression analysis (Proc REG, SAS Institute 1990) for the spray-dried formulations only. For this analysis, the 'stepwise selection' option was used to determine significant relationships between amounts of each ingredient (lignin, flour, TiO_2, and sugar) relative to the calculated LC_{50}. Calcium chloride was not included among the ingredients because it was added to the formulation at 10% of the weight of lignin (covariance = 1.00) and thus would give the same relationship as observed for lignin. Stepwise regression analysis included variables in the model in a stepwise fashion, and only if the variable was significant at $P < 0.15$ level (Proc Reg, SAS Institute 1990). Only variables with a significance of $P < 0.05$ are reported. This stepwise regression analysis was not run for data sets, which LC_{50} values were not determined by POLO-PC (LeOra Software 1987) for one or more of the formulations. A paired *t*-test was used to compare results for paired formulations made with and without flour, sugar or TiO_2 (Proc Means, SAS Institute 1990). For paired data, analysis consisted of comparing the difference between LC_{50} values (dosage response assays) or insect mortality (field experiments) for paired formulations and analyzing the difference for significance to zero (H_o: $\mu 1 - \mu 2 = 0$). LC_{50} values for formulations were subjected to analysis of variance (ANOVA) using production lot of virus as replications and means were separated by least significant difference (LSD) (Proc GLM, SAS Institute 1990).

Field Applications and Bioassay Procedures. Two field experiments, applied on 3 June and 15 June 1999, were completed in Peoria, IL, at NCAUR field plots. For both experiments, procedures were the same and used the same plants. Cabbage ('Bravo' F1 hybrid) was transplanted ≈1 mo before the first field experiment. Each plot was one row consisting of nine plants spaced ≈60 cm apart. Rows were spaced 1.2 m apart. Applications were made before sunrise with a CO_2-charged backpack sprayer rigged with three nozzles, one directed over the top and one on each side of the row. The sprayer was calibrated to deliver 160 liters/ha. Formulations were mixed to provide 2.5×10^{12} ob/ha. Ten formulations made with each of the three virus lots (30 formulation samples) and stored for 7 mo at 4°C, were applied to field plots such that each virus lot was applied to respective blocks in a randomized complete block design. In addition, a single fresh *Af*MNPV formulation [PC1307+ PCF 1000 flour (Lauhoff Grain Co., Danville, IL) + TiO_2], was made with just one lot of virus (lot B), and a commercial *Af*MNPV formulation (Glycerin + TiO_2) was provided by Certis USA. These additional two formulations were mixed once and applied to each of three plots in the above-described randomized complete block design. Cabbage leaves were collected 3, 7, 27, and 51 h after application. A time 0, sample was not taken to allow time for applications and dew to dry from the plants before sampling. For each collection, one leaf disk was removed from each of five middle plants in each plot and placed individually into 5-cm-diameter plastic Petri dishes containing a piece of filter paper. Approximately twelve *T. ni* neonates were added to each dish, which was then capped with a sealing lid and placed in a dark incubator at 28°C for a 48-h feeding period. After feeding, six live larvae from each dish (30 larvae per plot) were transferred to artificial diet and incubated additional 5 d before mortality was assessed.

Results

Initial Insecticidal Activity of *Af*MNPV Formulations. Spray drying generally did not cause significant ($P > 0.05$) loss of insecticidal activity for these experimental formulations when compared with the unformulated samples based on the results from droplet-feeding and cotton-leaf assays (time 0, see Tables 3 and 5). The only exception was the lignin formulation made with virus lot B, which had significantly greater LC_{50} (less activity) when compared with the unformulated sample stored frozen in the droplet-feeding assay (Table 3). However, 21 of the 24 experimental formulations had initial LC_{50} values that were numerically greater than the LC_{50} value for their corresponding unformulated samples. Thus, spray-dried formulations may have experienced a nominal loss of insecticidal activity due to the drying process, a loss that would require many replications to indicate statistical differences. LC_{50} values determined based on the droplet-feeding assays were subjected to one-way ANOVA for the main effect to compare among formulations and formulation means were separated by LSD. For comparisons among treatments, no formulation was significantly different from unformulated virus (0.91×10^5 ob/ml). However, the average LC_{50} for lignin (1.49×10^5 ob/ml) and lignin + TiO_2 (1.46×10^5 ob/ml) formulations were significantly (LSD = 0.59×10^5 ob/ml) greater than the average LC_{50} for the glycerin formulation (0.85×10^5 ob/ml). Virus lots were also compared based on one-way ANOVA and using formulations as replications. Mean LC_{50} values among virus lots were significantly different (LSD = 0.32×10^5 ob/ml, $P = 0.05$). The mean LC_{50} for formulations made with virus lot A (1.9×10^5 ob/ml) was greater than the means for lots B and C, 0.9 and 0.8×10^5 ob/ml, respectively, indicating that lot A had less initial activity.

Several important consistencies were observed over

Table 3. Insecticidal activity of *Af*MNPV ($LC_{50} \times 10^5$ ob/mL) in spray-dried formulations after storage (months) at 30°C based on LC_{50} of *Trichopluisa ni* neonates in a droplet bioassay

Formulation	Months of storage			
	0	1	3	6
Lot A				
Lignin	2.0	49.0*	72.0*	N.D.
Lignin + flour	1.7	5.0*	30.0*	N.D.
Lignin + TiO_2	2.3	13.0*	72.0*	N.D.
Lignin + flour + TiO_2	3.2	4.3*	27.0*	49.0*
Lignin + sugar	2.0	7.4*	23.0*	560.0*
Lignin + flour + sugar	1.8	4.9*	24.0*	570.0*
Lignin + 2 flour + sugar	2.3	6.0*	4.5	27.0*
K-Lignin + 2 flour + sugar	2.0	5.2*	5.9	30.0*
Glycerin	1.4	1.0	1.9	11.0*
Unformulated, frozen	1.3	0.4	1.7	0.8
Lot B				
Lignin	1.8	4.1*	43.0*	130.0*
Lignin + flour	1.0	3.1*	1.6	19.0*
Lignin + TiO_2	1.4	3.7*	5.9*	99.0*
Lignin + flour + TiO_2	0.6	1.2	2.6	14.0*
Lignin + sugar	0.6	1.1*	4.9	N.D.
Lignin + flour + sugar	1.1	1.3*	1.4	4.2*
Lignin + 2 flour + sugar	0.6	1.4*	0.8	23.0*
K-Lignin + 2 flour + sugar	0.6	1.8*	1.5	7.1*
Glycerin	0.5	0.3	0.1	2.2*
Unformulated, frozen	0.6	0.3	1.8	0.3
Lot C				
Lignin	0.7	2.0*	9.8*	390.0*
Lignin + flour	0.8	1.9*	4.6*	11.0*
Lignin + TiO_2	0.7	1.2	7.3*	11.0*
Lignin + flour + TiO_2	0.4	1.7*	6.2*	14.0*
Lignin + sugar	1.1	2.9*	6.5*	N.D.
Lignin + flour + sugar	0.5	1.1	2.3*	5.2*
Lignin + 2 flour + sugar	1.2	2.4*	6.2*	27.0*
K-Lignin + 2 flour + sugar	0.9	2.3*	3.6*	9.4*
Glycerin	0.3	0.2	1.6*	9.0*
Unformulated, frozen	0.9	0.6	0.4	0.2

Refer to Table 2 for formulation composition.

* Significantly different than the insecticidal activity of the corresponding lot of frozen unformulated *Af*MNPV. Based on POLO-PC computer program, confidence limits at 90%. Five dose per assay. N.D. = not determined.

Table 4. Insecticidal activity of *Af*MNPV ($LC_{50} \times 10^5$ obs/mL) in spray-dried formulations after storage (months) at 4°C based on LC_{50} of *Trichopluisa ni* neonates in a droplet bioassay

Formulation	Months of storage		
	6	24	30
Lot A			
Lignin	4.1	4.2	3.9
Lignin + flour	1.4	4.8	6.2
Lignin + TiO_2	2.5	4.5	5.3
Lignin + flour + TiO_2	2.5	5.9	5.1
Lignin + sugar	1.2	4.9	2.6
Lignin + flour + sugar	0.9	8.2	5.3
Lignin + 2 flour + sugar	1.1	11.8	4.2
K-Lignin + 2 flour + sugar	1.3	9.5	4.4
Glycerin	0.8	1.2	0.7
Unformulated, frozen	1.6	13.9	1.0
Lot B			
Lignin	0.4	0.8	3.2
Lignin + flour	0.5	1.3	5.1
Lignin + TiO_2	0.6	1.7	4.8
Lignin + flour + TiO_2	0.5	1.8	49.0
Lignin + sugar	0.4	1.7	5.0
Lignin + flour + sugar	0.4	2.4	3.1
Lignin + 2 flour + sugar	0.3	1.9	3.0
K-Lignin + 2 flour + sugar	0.4	1.1	3.1
Glycerin	0.7	0.2	0.2
Unformulated, frozen	1.1	0.9	1.7
Lot C			
Lignin	0.6	2.1	8.9
Lignin + flour	0.5	2.8	9.6
Lignin + TiO_2	0.2	2.5	6.9
Lignin + flour + TiO_2	0.3	2.8	122.0
Lignin + sugar	0.3	2.0	7.3
Lignin + flour + sugar	0.2	1.6	5.4
Lignin + 2 flour + sugar	0.2	1.0	4.6
Lignin + 2 flour + sugar	0.4	1.2	4.3
Glycerin	0.2	0.4	0.7
Unformulated, frozen	0.6	0.8	1.1

Refer to Table 2 for formulation composition.

* Significantly different than the insecticidal activity of the corresponding lot of frozen unformulated *Af*MNPV. Based on POLO-PC computer program, confidence limits at 90%. Five does per assay. N.D. = not determined.

the course of this study. First, the two laboratory-assay techniques showed similar results indicating loss of activity for the virus formulations. For example, when samples were stored at 30°C for 6 mo, they lost significant insecticidal activity compared with unformulated virus stored frozen based on the results of the droplet-feeding assay (Table 3). For these same comparisons, 18 of the 24 spray-dried samples lost significant activity based on the leaf-feeding assay (Table 5). Similar results from the two techniques support the concept that a loss of activity would be apparent under field applications. Second, frozen unformulated virus provided relatively consistent results among assays conducted over this storage period and demonstrated the relative consistency of the procedures (Tables 3–6). This consistency also demonstrates that the loss of activity observed for the formulated samples was real and not an artifact of changing assay efficiency. Finally, formulations made with lot A had less activity initially and generally lost activity faster than like samples made with lots B and C. This result was apparent from both assay techniques and, not only supports the consistency among the assays, but demonstrates the importance of the initial virus stock used to make the formulations.

Results from the droplet-feeding and leaf-feeding assays also had some differences. The leaf-feeding assay (Tables 3 and 4) provided higher LC_{50} values for each sample compared with the results from droplet-feeding assay (Tables 5 and 6). Also, the four lignin formulations without sugar tended to have higher LC_{50}s compared with the four lignin formulations with sugar in the droplet-feeding assays, although they had lower LC_{50}s in the leaf-feeding assays.

Regression analysis of the lignin formulations to relate ingredients with insecticidal activity (droplet assay results) did not indicate significant relationships ($P > 0.05$ for slope parameter) for freshly prepared samples. After 1-mo storage at 30°C, lignin concentration in the spray-dried formulations had a positive relationship (slope $= 0.66 \times 10^6$ ob/ml^{-1}·g^{-1}, $F = 6.99$, $P = 0.0148$) with insecticidal activity. A similar relationship was identified by regression analysis of the mortality data for samples stored 3 mo at 30°C. Lignin concentration in the formulation had a significant positive relationship with LC_{50} values (slope $= 1.56 \times 10^6$

Table 5. Insecticidal activity of *Af*MNPV ($LC_{50} \times 10^5$ obs/mL) spray-dried formulations after storage (months) at 30° based on LC_{50} of *Trichopluisa ni* neonates in a cotton-plant bioassay

Formulation	Months of storage		
	0	4	6
Lot A			
Lignin	5.8	N.D.	N.D.
Lignin + flour	6.5	4.1	100.0*
Lignin + TiO_2	1.4	4.6	46.0*
Lignin + flour + TiO_2	6.0	3.1	43.0*
Lignin + sugar	5.3	N.D.	N.D.
Lignin + flour + sugar	10	N.D.	51.0*
Lignin + 2 flour + sugar	1.9	N.D.	1000.0*
K-Lignin + 2 flour + sugar	3.1	N.D.	71.0*
Glycerin	7.5	3.8	52.0*
Unformulated, frozen	6.0	0.3	1.4
Lot B			
Lignin	2.1	3.9	51.0*
Lignin + flour	0.6	5.2	14.0*
Lignin + TiO_2	0.3	2.3	6.4*
Lignin + flour + TiO_2	1.5	3.9	1.6
Lignin + sugar	1.1	N.D.	330.0*
Lignin + flour + sugar	1.2	N.D.	2.7
Lignin + 2 flour + sugar	3.2	N.D.	8.8*
K-Lignin + 2 flour + sugar	1.5	N.D.	3.5
Glycerin	1.6	5.9	14.0*
Unformulated, frozen	2.9	2.3	1.0
Lot C			
Lignin	0.4	5.0	2.3
Lignin + flour	0.9	5.2	1.3
Lignin + TiO_2	0.7	6.5	47.0*
Lignin + flour + TiO_2	7.6	6.5	9.1*
Lignin + sugar	1.6	N.D.	41.0*
Lignin + flour + sugar	2.9	N.D.	7.4
Lignin + 2 flour + sugar	0.8	N.D.	10.0*
K-Lignin + 2 flour + sugar	2.1	N.D.	8.9*
Glycerin	0.6	4.1	71.0
Unformulated, frozen	1.2	5.2	2.0

Refer to Table 2 for formulation composition.

* Significantly different than the insecticidal activity of the corresponding lot of frozen unformulated *Af*MNPV. Based on POLO-PC computer program, confidence limits at 90%. Five does per assay. N.D. = not determined.

Table 6. Insecticidal activity of *Af*MNPV ($LC_{50} \times 10^5$ obs/mL) spray-dried formulations after storage (months) at 4° based on LC_{50} of *Trichopluisa ni* neonates in a cotton-plant bioassay

Formulation	Months of storage	
	6	30
Lot A		
Lignin	30.0	3.4
Lignin + flour	10.0	2.3
Lignin + TiO_2	2.8	3.2
Lignin + flour + TiO_2	3.8	1.8
Lignin + sugar	4.8	1.8
Lignin + flour + sugar	6.9	2.5
Lignin + 2 flour + sugar	3.4	2.6
K-Lignin + 2 flour + sugar	4.2	0.7
Glycerin	4.0	0.8
Unformulated, frozen	3.0	1.0
Lot B		
Lignin	2.8	2.2
Lignin + flour	1.6	1.6
Lignin + TiO_2	3.4	1.5
Lignin + flour + TiO_2	1.9	2.2
Lignin + sugar	1.6	1.6
Lignin + flour + sugar	1.5	1.6
Lignin + 2 flour + sugar	1.7	1.3
K-Lignin + 2 flour + sugar	1.0	3.0
Glycerin	1.0	0.9
Unformulated, frozen	3.7	1.3
Lot C		
Lignin	2.0	2.6
Lignin + flour	0.8	4.1
Lignin + TiO_2	0.8	6.3
Lignin + flour + TiO_2	0.9	3.5
Lignin + sugar	1.1	2.6
Lignin + flour + sugar	1.5	2.0
Lignin + 2 flour + sugar	1.5	1.5
K-Lignin + 2 flour + sugar	0.9	1.2
Glycerin	0.5	1.6
Unformulated, frozen	1.6	3.4

Refer to Table 2 for formulation composition.

* Significantly different than the insecticidal activity of the corresponding lot of frozen unformulated *Af*MNPV. Based on POLO-PC computer program, confidence limits at 90%. Five does per assay. N.D. = not determined.

$ob/ml^{-1}g^{-1}$, $F = 9.22$, $P = 0.0061$). After storage for 6 mo, six formulations lost insecticidal activity to a point where the dosage response data did not fit probit analysis and their LC_{50} values were reported as not determined (ND) in Table 3.

Samples of spray-dried formulations stored at 4°C retained insecticidal activity better than samples stored at 30°C. After 6 mo of storage at 4°C (Table 4), insecticidal activity of experimental formulations was comparable with the frozen unformulated control based on overlapping confidence intervals. After 30 mo of storage, 9 of 24 experimental formulations had significantly higher LC_{50s} compared with their respective unformulated virus samples, which had been stored frozen. Regression analysis to relate ingredient concentration with loss of insectical activity after 6 mo and 30 mo of storage did not indicate a significant ($P > 0.05$) relationship between ingredients and insecticidal activity determined by the droplet-feeding assay.

Plant-feeding assays of stored samples showed some similarities with droplet-feeding assays in that formulations stored at 30°C lost significant insecticidal activity within 6 mo (Table 5). Eighteen of the 24 experimental formulations had significantly greater LC_{50} values compared with the unformulated samples stored frozen based on nonoverlapping confidence limits (90%) (Table 5). For samples stored at 4°C (Table 6), no significant differences were observed when stored for 6 or 30 mo.

Regression analysis to relate individual formulation ingredients to insecticidal activity based on the leaf-feeding assay showed no significant trends for freshly made experimental formulations. Thus, the lignin-based spray-dried formulation is robust and will allow the inclusion of a variety of ingredients without an initial loss of insecticidal activity. After 6 mo of refrigerated storage, the concentration of lignin showed a positive relationship with LC_{50} values (slope = 0.38×10^6 $ob/ml^{-1}g^{-1}$, $F = 5.94$, $P = 0.0234$) indicating that formulations with more lignin were likely to lose insecticidal activity during storage. After 30 mo of storage, sugar content showed a negative relationship with the LC_{50} values (slope = -0.08×10^6 $ob/ml^{-1}g^{-1}$, $F = 5.07$, $P = 0.0347$) of the experimental formulations. Thus, formulations with higher sugar content

Table 7. Average percentage mortality of neonate *Trichoplusia ni* after feeding on samples of field-grown cabbage leaves treated with formulations of *Af*MNPV, applied June 3, 1999

Treatments[a]	Hours after application when leaves were sampled[b]		
	3	7	27
Control	2.2a	2.2a	1.0a
Unformulated *Af*MNPV	29.8b	18.9ab	6.7ab
Lignin	67.8cd	70.0de	30.0bcd
Lignin + flour	56.7c	26.8b	28.9bcd
Lignin + TiO_2	82.2cd	78.9e	37.0cde
Lignin + flour + TiO_2	57.8c	56.0cde	38.9cde
Lignin + sugar	85.4d	74.4e	61.1e
Lignin + flour + sugar	83.1cd	67.8de	54.4e
Lignin + 2 flour + sugar	75.6cd	62.2de	46.7de
K-Lignin + 2 flour + sugar	66.7cd	72.2de	50.0de
Lignin + flour + TiO_2 (fresh)	80.0cd	69.6de	55.6e
Glycerin formulation	60.0cd	50.8cd	16.7abc
Glycerin formulation[c] + TiO_2	71.1cd	32.8bc	29.2bcd
F	6.5	9.2	5.1
SEC	13.1	11.5	11.8
CVC	26.8	23.5	24.2

[a] Refer to Table 2 for formulation compositions. Treatments were applied at a rate of 2.5×10^{12} ob/Ha, except for the untreated control.
[b] Means in a column followed by the same letter are not significantly different, LSD ($P < 0.05$).
[c] Glycerin formulation + TiO_2, was supplied by Certis USA, Columbia, MD.

tended to have lower LC_{50} values (higher insecticidal activity) after extended storage.

Preplanned comparisons (paired *t*-test) of specific formulations with and without ingredients such as flour, sugar, or TiO_2 showed few consistent differences among the evaluations of the experimental results. Based on droplet assays, lignin formulations with flour had significantly lower LC_{50}s (difference = -16.1×10^5 ob/ml, SE = 6.71, $t = 2.39$, $P = 0.044$) than similar formulations made without flour after storage for 3 mo at 30°C. Likewise, formulations made with sugar averaged significantly lower LC_{50}s (difference = -4.56×10^5 ob/ml, SE = 0.85, $t = 5.37$, $P = 0.003$) than similar formulations made without sugar after storage for 30 mo at 4°C. None of the other paired comparisons for like formulations made with or without flour, sugar or TiO_2 were significantly different ($P > 0.05$). For the plant-feeding bioassays, none of the paired comparisons showed significant ($P > 0.05$) differences for comparing formulations made with or without flour, sugar or TiO_2. Results indicate that these formulation ingredients impart subtle effects on the storage stability of the spray-dried formulations.

Field Experiments. For the field experiment applied on 3 June, a rain event (4.5 cm) was recorded between 33 and 40 h after application. This rain washed treatments from the plants and resulted in the low mortalities (<10% mortality) for nearly all treatments collected 51 h after application, and the 51-h data are not recorded in Table 7. Untreated controls (no virus application) averaged a mortality of 1.8%. Few significant differences were observed among lignin-based formulations in this field experiment (Table 7). Unformulated *Af*MNPV lost significant insecticidal activity within 3 h of application when compared with treatments of formulated virus. Glycerin formulations (with or without TiO_2) showed higher activity compared with the unformulated virus treatment. Adding TiO_2 to the glycerin formulation did not provide any benefit, as the two treatments were not significantly different ($P > 0.05$). As in the first experiment, unformulated *Af*MNPV lost significant insecticidal activity after 3 h of application with <30% mortality. More significant differences were observed among formulations in the second field experiment (Table 8) compared with the first experiment. No rainfall was recorded during the second field experiment applied 15 June. As in the first experiment, untreated controls (no virus application) averaged a low mortality of 7.8%.

Table 8. Average percentage mortality of neonate *Trichoplusia ni* after feeding on samples of field grown cabbage leaves treated with formulations of *Af*MNPV, applied June 15, 1999

Treatments[a]	Hours after application when leaves were sampled[b]			
	3	7	27	51
Control	19.0a	1.1a	3.3a	3.4a
Unformulated *Af*MNPV	30.0ab	23.5abc	15.5ab	11.3ab
Lignin	42.3abc	14.2ab	30.1bcde	22.2abc
Lignin + flour	50.0bc	45.6cde	34.7bcde	25.6abc
Lignin + TiO_2	65.6def	45.6cde	42.2cde	27.8bc
Lignin + flour + TiO_2	52.2bcd	56.4def	35.2bcde	22.4abc
Lignin + sugar	63.3def	61.1ef	55.8e	32.2bc
Lignin + flour + sugar	65.6def	77.8f	29.4abcd	35.6cd
Lignin + 2 flour + sugar	75.6ef	62.7ef	30.0bcde	28.9bc
K-Lignin + 2 flour + sugar	81.1f	61.1ef	55.6de	56.7d
Lignin + flour + TiO_2 (fresh)	64.2def	62.9ef	42.2cde	36.7cd
Glycerin formulation	56.7def	27.8abcd	27.2abc	16.7abc
Glycerin formulation[c] + TiO_2	56.1de	41.1bcde	20.0abc	10.4ab
F	4.1	4.4	2.7	3.0
SEC	12.1	15.1	12.7	11.2
CVC	24.9	31.0	26.2	23.1

[a] Refer to Table 2 for formulation compositions. Treatments were applied at a rate of 2.5×10^{12} ob/ha, except for the untreated control.
[b] Means in a column followed by the same letter are not significantly different, LSD ($P < 0.05$).
[c] Glycerin formulation + TiO_2, was supplied by Certis USA, Columbia, MD.

Field applications showed no advantage for the freshly-made formulation when compared with formulations that had been stored for over 6 mo under refrigeration (Tables 7 and 8). Overall, experimental formulations made with lignin in combination with sugar, alone or in combination with other ingredients, showed the best residual activity in both field experiments.

Comparisons of insect mortality from field data for formulations made with flour compared with like formulations made without flour showed no significant ($P > 0.05$) difference, indicating no advantage or disadvantage for formulations made with flour. For the first field experiment, formulations made with sugar averaged 24% greater mortality (SE = 5.06, $t = -4.81$, $P = 0.005$) than the like formulations made without sugar. In the second experiment, the comparison of paired data did not indicate a significant advantage for formulations that had sugar as a formulation ingredient. In both experiments, formulations with TiO_2 averaged significantly (first experiment difference = 11.8%, SE = 3.92, $t = -3.01$, $P = 0.030$; second experiment difference = −10.3%, SE = 4.18, $t = -2.47$, $P = 0.042$) higher insect mortality compared with similar formulations made without TiO_2 indicating an advantage for extending residual insecticidal activity by adding TiO_2 to the lignin-based dried formulations.

Discussion

Formulation development is important to maximize economic characteristics of biological pesticides to promote commercialization. Some characteristics that improve product economics include high product efficacy, low cost of ingredients, storage stability, and simple or common commercial production techniques. Formulation research presented here addressed storage stability and field efficacy of lignin-based baculovirus insecticides. The ingredients used to produce these formulations were selected based on previous research for extended residual activity (Tamez-Guerra et al. 2000, McGuire et al. 2001) virus encapsulation, and storage stability (Tamez-Guerra et al. 2002).

Baculoviruses have remained active after 15 yr of storage (Steinhaus 1954). Bergold (1958, cited in Lewis and Rollinson 1978) reported that polyhedra of *Bombyx mori* (L.) lost little or no activity after 9 yr. In contrast to these reports, Cunningham (1970) demonstrated a loss of insecticidal activity for a baculovirus of the hemlock looper, *Lambdina fiscellaria fiscellaria* (Guenee), stored for 6 yr as an aqueous suspension under refrigerated conditions, and suggested that freeze-drying may be better for storing virus. Vail et al. (1991) demonstrated a greater loss of insecticidal activity for a granulosis virus after storage for 1–3 mo at 32° or 38°C when compared with 27°C. Dry virus has been shown to lose activity during storage at room temperatures (Lewis and Rollinson 1978) and at cold temperatures (Kaupp and Ebling 1993). However, retaining a measurable level of insecticidal activity is not necessarily consistent with preventing a significant loss of activity for a commercial biopesticide. These experiments demonstrated that virus formulations stored at warm temperatures (30°C) lost significant insecticidal activity. However, samples of the same formulations stored at refrigerated temperatures for more than two years did not lose activity. Previous research with similar virus formulations (Tamez-Guerra et al. 2002) showed reduced activity after storage at room temperatures for 1 yr. Medugno et al. (1997) reported no loss of activity for three of four formulations of *Anticarsia gemmatalis* polyhedrosis virus prepared by spray drying with clays and stored at room temperature for 1 yr. Thus, higher storage temperatures seem well correlated with reduced insecticidal activity of baculovirus for both unformulated and formulated samples. However, refrigerated temperature is apparently adequate to maintain virus activity for extended storage of some formulations and indicates that frozen storage may not be necessary for many practical situations including commercial production.

Specific comparisons to relate formulation ingredients with insecticidal activity indicated a few trends to consider for further optimization of virus formulations. Concentrations of various ingredients were not related to variability in insecticidal activity for freshly made formulations, and thus allows the selection of formulation ingredients based on benefits offered to other factors such as cost, storage stability, and/or field activity. Related research identified spray-drying conditions that reduced initial insecticidal activity during production such as pH of the dryer feedstock and use of alternative lignins (R. Behle, P. Tamez-Guerra, and M. McGuire, unpublished data), conditions that were avoided during production of these formulations. After storage at 30°C for 1 and 3 mo, formulations made with more lignin tended to have higher LC_{50} values (droplet-feeding assays), suggesting that adding more lignin reduces the storage stability of virus formulations. This relationship was confirmed by the plant-feeding assay of formulations stored for 6 mo in the refrigerator. The only other relationship identified by regression analysis was a reduction in LC_{50} values with increased sugar content indicating that including sugar as a formulation ingredient benefits insecticidal activity. This relationship agrees with observations reported by Tamez-Guerra et al. (2002) that dried virus formulations made with some sugar retained insecticidal activity during storage better than similar formulation with no sugar. Paired comparisons of formulations in some data sets showed benefits of including both flour and sugar as part of the lignin-based formulation. Combining these observed relationships to maximize storage stability of the virus formulations should lead to formulations made with less lignin and more sugar and flour, until other characteristics such as ultraviolet protection are compromised.

Two types of assays were used to measure different characteristics of the formulations. The droplet assay measured only insecticidal activity and does not take into account other factors such as feeding preference,

effect of drying, or formulation x leaf interactions. The leaf-feeding assay should more closely resemble a field situation. These data suggest that formulations containing sugar had higher activity based on the leaf-feeding assay. There is a precedence for adding sugar to improve palatability of formulations of *Bacillus thuringiensis* (Bell and Romine 1980, Bartelt et al. 1990). Williams et al. (1999) added sugar as a formulation ingredient for a baculovirus application to control *Spodoptera frugiperda*, but concluded that sugar did not act as a feeding stimulant in two field trials. Adding sugar to the formulation may protect virus activity during transition from wet to dry (Behle et al. 2000), such as after spray application. Sugar may have many other affects that have yet to be identified. Our data do not identify how the addition of sugar improved the spray-dried formulations. The droplet-feeding assay maintains an aqueous suspension of virus in a relatively concentrated sucrose solution (2% wt:vol) effectively removing both factors (drying and feeding preference), which could influence insecticidal activity in other assays. In contrast, the leaf-feeding assay allows the virus suspension to dry on the leaf surface and could improve insecticidal activity by protecting the virus while drying or by inducing preferential feeding on the treated portions of the leaf. Regardless of the specific mechanism of activity, our results support the additions of sugar to virus formulations.

Field application of these formulations evaluated a different set of considerations for formulation efficacy. Generally, the lignin-based formulations showed few differences among themselves for insecticidal activity when applied under field conditions, but lignin formulations did protect the residual activity of the virus longer compared with the unformulated and glycerin based formulations. Field experiments indicated a benefit for adding TiO_2 to lignin-based formulation. Paired evaluations comparing similar formulations (with versus without TiO_2) showed an advantage for including this ingredient and may have resulted from extending the residual activity of these formulations. This finding agrees with Bull et al. (1976), who reported the benefit of including TiO_2 as a formulation ingredient to prevent virus inactivation when exposed to ultraviolet irradiation.

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